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## Presence of EBV DNA in Cerebrospinal Fluid is Associated with Greater HIV RNA and Inflammation

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(Article begins on next page)

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Running Head: EBV and HIV in CSF

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46 **Abstract**

47

48 Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) can infect several cells, replicate in the  
49 central nervous system and affect blood-brain barrier (BBB) integrity. This study aimed to  
50 investigate whether cerebrospinal fluid (CSF) EBV or CMV DNA was associated with viral,  
51 inflammatory and neuronal damage biomarkers in people living with HIV (PLWH).

52 EBV, CMV DNA and HIV RNA were measured on CSF, through RT-PCR, from PLWHs  
53 undergoing lumbar punctures for clinical reasons (excluding onco-haematological  
54 comorbidities). Immune-enzymatic assays evaluated BBB inflammation and damage. Patients  
55 were stratified according to plasma HIV RNA levels in viremic ( $\geq 50$  copies/mL) and aviremic  
56 ( $< 50$  copies/mL). We included 298 participants. Among 167 viremic patients, CSF EBV and  
57 CMV DNA were detectable in 42 (25.1%) and 10 (6.3%) participants; among 130 aviremic  
58 subjects CSF EBV and CMV DNA were detectable in 12 (9.2%) and 0 (0%) participants,  
59 respectively. In viremic group, detectable CSF EBV DNA was associated with CSF pleocytosis  
60 ( $p < 0.001$ ), higher CSF HIV RNA ( $p < 0.001$ ) and neopterin levels ( $p = 0.002$ ). In aviremic  
61 participants, detectable EBV DNA was associated with pleocytosis ( $p = 0.056$ ), higher neopterin  
62 ( $p = 0.027$ ) and immune globulins ( $p = 0.016$ ) in the CSF; CSF escape was more common in those  
63 with detectable EBV DNA (50% vs. 21.2%,  $p = 0.036$ ).

64 EBV DNA was frequently detected in the CSF of viremic and fewer aviremic patients on  
65 antiretroviral treatment. In PLWH without clinical evidence of encephalitis CSF EBV DNA was  
66 associated with higher levels of HIV RNA and biomarkers of neuronal damage/inflammation.  
67 The role of EBV reactivation in HIV-associated CNS disorders warrants further studies.

68

ha eliminato:

ha eliminato: The aim of this study was

ha eliminato: We examined CSF samples participants undergoing lumbar punctures for clinical reasons (excluding those with lymphoproliferative disorders): we measured EBV, CMV DNA and HIV RNA (by PCR), markers of neuronal damage and inflammation (by immune-enzymatic assays).

ha eliminato: (with plasma HIV RNA  $< 50$  copies/mL)

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ha eliminato: EBV DNA was frequently detected at low levels in the CSF of viremic participants and in a minority of aviremic patients on antiretroviral treatment.

## Background:

The central nervous system (CNS) is a clinically relevant target of HIV and severe forms of encephalitis and dementia have been described since the beginning of the epidemics [1]. Even in the current era of antiretroviral therapy (ART), chronic CNS involvement is a significant issue for people living with HIV (PLWH) [2]. In fact, HIV persists in the brain tissue of people living with HIV (PLWH) despite systemic viral control and its detection in the cerebrospinal fluid (CSF) has been associated with acute/subacute neurological symptoms, worse neurocognitive performances and immune activation [3]. While the clinical relevance of HIV RNA escape within the CSF is controversial, symptomatic cases have been described and ART optimizations have led to clinical/radiological/virologic improvements, thus supporting a pathogenetic role of HIV RNA active replication in the CNS [4]. The factors associated with HIV RNA escape in the CSF are poorly understood with several identified risk factors, such as low nadir CD4 cell count, dementia, poor adherence to ART, low level viremia in blood plasma and the presence of drug resistance associated mutations [5-6]. Another possible driver of compartmentalized HIV RNA replication in the CSF might be co-infection with other chronic viruses ("secondary escape") but this has not been systematically investigated [7].

As part of this study, we focused on the effects of Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) since PLWH have a higher risk of acquiring and incompletely controlling both viruses; both EBV [8-10] and CMV [11-13] have been associated with neurological disorders as well as vascular inflammation, thus suggesting the potential for chronic CNS and endothelial involvement (well recognized for CMV) [11-13]. In particular, EBV can infect macrovascular endothelial cells in human tissue [14-17], human brain micro-vessels [18] and human umbilical vein endothelial cells [19]. Endothelial cells with lytic reactivation of EBV present increase

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129 production of pro- inflammatory molecules (CCL-2 and CCL-5) and also hyper-expression of  
130 surface adhesion molecules (ICAM-1 and VCAM-1) with a potential creation of an inflammatory  
131 breach through the Blood Brain Barrier (BBB) [12, 20-21]. This may be relevant because a key  
132 factor in chronic HIV RNA CNS involvement seems to be the alteration of the BBB: the latter  
133 has been recently described as being part of the neurovascular unit where endothelial cells  
134 (and pericytes) co-operate with astrocytes and neurons [22]. Thus, CMV and EBV may  
135 potentially cause a sub-clinical chronic infection and facilitate inflammatory cells' trafficking  
136 through the BBB increasing migration of HIV into the CNS. [7, 15, 23]

137

## 138 **Material and Methods**

### 139 **Cohort and Samples**

140 We enrolled adult PLWH undergoing lumbar punctures for clinical reasons, [in a cross-sectional](#)  
141 [design study](#), including late presentation with <100 CD4<sup>+</sup> T lymphocytes/mm<sup>3</sup> [in peripheral](#)  
142 [blood](#), opportunistic infections, new or persistent neurological symptoms (including headache),  
143 worsening cognitive impairment, need of lumbar puncture in case of syphilis or white matter  
144 hyperintensities at brain MRI. Patients with primary central nervous system lymphomas,  
145 lymphoproliferative diseases and autoimmune disorders were excluded from this study. [We](#)  
146 [also excluded HIV controllers without ART.](#) Demographic, immunovirological, clinical and  
147 therapeutic data were recorded as well as CSF characteristics. The protocol was approved by  
148 our Ethics Committee (Comitato Etico Inter-aziendale di Orbassano, n. 103/2015). Study  
149 participants signed a written informed consent at enrollment.

### 150 **Viral Measures**

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152 Levels of HIV RNA were measured by real time Polymerase Chain Reaction (RT-PCR) assay  
153 CAP/CTM HIV-1 vs. 2.0 (CAP/CTM, Roche Molecular System, Branchburg, NJ, HIV RNA  
154 detection limit: 20 copies/mL). Levels of EBV DNA and CMV DNA were measured through the  
155 RT-PCR (detection limit: 100 copies/mL). CSF escape was defined as CSF HIV RNA >50  
156 copies/mL with plasma HIV RNA <50 copies/mL and CSF/plasma discordance as CSF HIV  
157 RNA 0.5 Log<sub>10</sub> higher than plasma HIV RNA (if both were detectable) [24].

#### 158 **Immunological Measures and BBB Damage**

159 Quantitative determination of albumin in serum and CSF was measured by  
160 Immunospectrometric methods (AU 5800, Beckman Coulter, Brea, CA, USA). CSF to serum  
161 albumin ratio (CSAR) was calculated as albumin in CSF (mg/L)/albumin in serum (g/L), and  
162 was used to evaluate BBB permeability. Impaired BBB was defined according to age-adjusted  
163 Reibergrams (normal if below 6.5 in patients aged <40 years and below 8 in patients >40 years)  
164 [25]. The presence of Immune globulins (Ig)G produced inside the CNS was calculated  
165 according to Tibbling index [26]. CSF pleocytosis was defined as  $\geq 5$  cells/mm<sup>3</sup>.

166 CSF total tau (t-tau), phosphorylated tau (p-tau) and 1-42  $\beta$ -amyloid ( $A\beta^{1-42}$ ) were measured by  
167 immunoassay methods (Fujirebio diagnostics, Malvern, U.S.A.) with limits of detection of  
168 57, 20 and 225 pg/ml, respectively. Neopterin and S100B were measured through ELISA [DRG  
169 Diagnostics (Marburg, Germany) and DIAMETRA S.r.l. (Spello, Italy), respectively]. Upper  
170 limits of normality in HIV-negative individuals were as follows: t-tau [ $<300$  pg/mL (in participants  
171 aged 21–50),  $<450$  pg/mL (in participants aged 51–70) or  $<500$  pg/mL in older participants], p-  
172 tau ( $<61$  pg/mL), 1–42 beta amyloid ( $>500$  pg/mL), neopterin ( $<1.5$  ng/mL) and S100B ( $<380$   
173 pg/mL) [27].

174

175 **Statistical Analysis**

176 We performed descriptive statistics on the entire study population and then stratified between  
177 study participants with suppressed plasma HIV RNA (<50 copies/mL) and those with detectable  
178 HIV RNA.

179 Data were analyzed using standard statistical methods: variables were described with medians  
180 [interquartile ranges (IQR)], groups were compared using non-parametric tests (Mann–Whitney,  
181 Kruskal-Wallis and Spearman’s tests as specified in the text). Linear logistic regressions were  
182 used for estimating the association between detectable EBV/CMV DNA, HIV RNA, as well as  
183 biomarker of CNS damage and inflammation. Models were adjusted for CD4<sup>+</sup> cell counts and  
184 CSF HIV RNA. Data analysis was performed using PASW software version 22.0 (IBM).

185

186 **Results**

187 Two hundred and ninety-seven PLWH were included in this study, of whom 118 (39.4%) were  
188 naïve for ART. Baseline and immune-virological characteristics, stratified by plasma HIV RNA  
189 (below or above 50 copies/mL) are shown in **Table 1**.

190 EBV DNA was detected in the CSF of 42 (25.1%) and 12 (9.2%) participants with detectable  
191 and undetectable HIV RNA in plasma, respectively (p<0.001). Similarly, higher levels of EBV  
192 DNA were observed in the CSF of participants with detectable plasma HIV RNA (152 vs. <100  
193 copies/mL, p<0.001). Virological, neuronal damage and inflammation biomarkers stratified by  
194 plasma HIV RNA and CSF EBV DNA detection are shown in **Table 2**.

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ha spostato in basso [1]: CMV DNA was detected only in the CSF of participants with plasma HIV RNA >50 copies/mL [10 (6.3%) vs. 0 (0%), p=0.006]; participants with detectable CSF CMV DNA presented low CD4<sup>+</sup> T lymphocytes (25 vs. 91/mm<sup>3</sup>; p=0.005), higher plasma and CSF HIV RNA (5.8 vs. 5.1 Log<sub>10</sub> copies/mL, p=0.039 and 5.0 vs. 3.6 Log<sub>10</sub> copies/mL, p=0.016), higher CSAR (9.0 vs. 5.6, p=0.046) and neopterin (5.4 vs. 2.2 ng/mL, p=0.012) and they were more often diagnosed with opportunistic infections (40% vs. 17.3%, p=0.093).<sup>†</sup>



208 CMV DNA was detected only in the CSF of participants with plasma HIV RNA >50 copies/mL  
209 [10 (6.3%) vs. 0 (0%), p=0.006]; participants with detectable CSF CMV DNA had lower CD4<sup>+</sup> T  
210 lymphocytes (25 vs. 91/mm<sup>3</sup>, p=0.005), higher plasma and CSF HIV RNA (5.8 vs. 5.1 Log<sub>10</sub>  
211 copies/mL, p=0.039 and 5.0 vs. 3.6 Log<sub>10</sub> copies/mL, p=0.016), higher CSAR (9.0 vs. 5.6,  
212 p=0.046) and neopterin (5.4 vs. 2.2 ng/mL, p=0.012) and they were more often diagnosed with  
213 opportunistic infections (40% vs. 17.3%, p=0.093).

ha spostato (inserimento) [1]

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215 In PLWH with detectable plasma HIV RNA, presence of detectable EBV DNA was associated  
216 with a significantly higher number of cells, greater CSAR, as well as increased HIV RNA and  
217 neopterin in the CSF. In participants with plasma HIV RNA <50 copies/mL, presence of  
218 detectable EBV DNA was associated with significantly higher number of cells and greater  
219 neopterin in the CSF. The presence of IgG produced within the CNS was more common in  
220 aviremic participants with detectable CSF EBV DNA (38% vs. 0%, p=0.036). Additionally, CSF  
221 HIV RNA escape was more common in ART-suppressed participants with detectable EBV DNA  
222 (50% vs. 21%, p=0.036) (Figure 1).

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223 No correlation was observed between EBV DNA concentrations and the studied biomarkers.

224 Linear logistic analysis (adjusted for CD4 cell count and CSF HIV RNA) suggested that a  
225 detectable EBV DNA was independently associated with CSF pleocytosis in PLWH with CSF  
226 HIV RNA <50 copies/mL (p<0.001) and non-controllers (p<0.001), with neopterin in HIV non-  
227 controllers (p=0.015) and with the production of IgG within the CNS in HIV-controllers (p=0.008).

228

232

## 233 Discussion

234 To better understand the role of EBV and CMV in the CNS on HIV RNA replication, BBB  
235 damage and biomarkers of neuronal damage/inflammation, we measured levels of CMV and  
236 EBV DNA in CSF of 298 PLWH.

237 Overall, we observed that EBV DNA was detectable at low levels in 18% of all PLWH and it  
238 was associated with higher levels of HIV RNA in the CSF and up to three-time higher rate of  
239 pleocytosis. Compared to viremic subjects (25.1%), EBV DNA was found less frequently in  
240 study participants with undetectable HIV RNA in plasma (9.2%), and its presence was  
241 associated with pleocytosis, IgG production within the CNS and presence of CSF HIV RNA  
242 escape. CMV DNA, on the contrary, was found only in HIV viremic participants and was  
243 associated with low CD4<sup>+</sup> cell count, high plasma HIV RNA and opportunistic disorders.

244 Our findings are similar to those reported by Weinberg et al. in HIV-negative individuals where  
245 the presence of pleocytosis was associated with detectable CSF EBV DNA but also presence  
246 of EBV related-mRNA, supporting the hypothesis that EBV DNA is not just carried by latently  
247 infected inflammatory cells (e.g. B cells) but the consequence of actively replicating virus [28].  
248 Furthermore, EBV affects the immune system and it may enhance neuronal degeneration in  
249 chronic inflammatory conditions [29-30]. Our data are in line with this hypothesis since both  
250 viremic and ART-suppressed PLWH [showed](#) higher CSF HIV RNA levels (and CSF to plasma  
251 HIV RNA ratios) and increased white blood cells when EBV DNA was detectable. Additionally,  
252 PLWH with detectable HIV RNA and EBV DNA in CSF also showed higher CSAR supporting  
253 a potential role of chronic EBV infection in BBB damage, which in turn is associated with

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255 neurocognitive impairment, and with neuronal damage and inflammation [31-33]. In vitro  
256 experiments suggest that hosting EBV astrocytes and microglia may enhance cell-to-cell  
257 crosstalk and favoring migration of monocytic/macrophagic line cells into the CNS [34-36]. This  
258 effect may be independent from HIV control and immune system improvement: these conditions  
259 have been associated with the absence of neuronal damage and with the lowest CSF  
260 concentrations of neopterin [37-39].

261 Despite the low level or absent plasmatic EBV and CMV replication can be found at higher  
262 concentrations in tissues and organs. In a recent study that analyzed 108 gut biopsies collected  
263 from 19 HIV-infected and 22 HIV-uninfected participants, CMV and EBV were detected in more  
264 than 70% of samples but more commonly in HIV-positive subjects [40]. While the negative  
265 effects of sporadic or continuous CMV replication are well-known, there is still uncertainty on  
266 the role of EBV in favoring chronic immune activation. Neuroinflammation, neurodegeneration  
267 and its drivers are widely studied in MS and Late EBV infection seemed to be one of the risk  
268 factors involved in promoting the initial events and the relapses of this chronic neurological  
269 condition [41-42]. In most CSF of MS patients were founded elevated antibody levels against  
270 the entire EBV nuclear antigen (EBNA), and EBNA-1, a protein expressed during latent EBV-  
271 infection [43-44]. Anti-EBNA-1 IgG antibodies were correlated also with CSF oligoclonal bands  
272 and in some patients oligoclonal bands include anti-EBV antibodies [45]. Reactivation of EBV  
273 in the central nervous system (CNS) has been proposed as a possible cause of MS although  
274 the virus has not been consistently found in MS lesions [41].

275 From a broader perspective the lifelong presence of most Herpesviridae in the organism may  
276 produce in some hosts alterations in neuronal cellular processes [46-47]. These observations  
277 were suggested by several studies suggesting an association between EBV, human

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279 herpesvirus-1 and 6 (HSV-1 and HHV-6) with Alzheimer's (AD) and other neurodegenerative  
280 diseases [48-50]. Very recently HHV-6A and HHV-7 produced disruption of molecular, genetic,  
281 and clinical networks was reported in autaptic brains from patients suffering of AD along with  
282 [51-52].

283 Additionally, EBV may play a role in suppressing the CNS immune system and therefore  
284 maintain an incomplete T-cell mediated inflammatory response, through the expression of viral  
285 genes encoding for proteins with immunoevasion-like function. This may translate into higher  
286 rates of pleocytosis but with less inflammatory activity [53-54]. On the other hand, ART-  
287 suppressed study participants with detectable EBV DNA showed lower CD4<sup>+</sup> T cell counts thus  
288 suggesting that immune control may be needed in order to restore a partial control on EBV  
289 replication.

290 CMV DNA was detected in naïve patients only and, specifically, in patients presenting with  
291 advanced immune depletion and opportunistic infections and with high viral replication both in  
292 plasma and CSF. Additionally, BBB damage and monocyte-derived inflammation were  
293 significantly higher in participants with detectable CSF CMV DNA. Further speculations on the  
294 role of CMV are limited by the low number of participants in this subgroup; yet it may be peculiar  
295 of individuals with very severe immunodeficit and, as already shown by several reports, at  
296 higher risk of poor survival. [55,56]

297 Some limitations of this study should be acknowledged including the low sample size, the cross-  
298 sectional design, the lack of a control group and the lack of plasma EBV DNA measurements.  
299 We also did not collect neurocognitive data in all participants. Importantly, in this observational  
300 study we cannot assess the causal relationship between presence of EBV, inflammation and  
301 HIV RNA escape. Additionally, our cohorts include several patients with very low nadir CD4<sup>+</sup> T

cell counts and heterogeneous clinical conditions: the same effect may not be observed in individuals with less advanced disease.

In conclusion we reported for the first time the presence of detectable EBV DNA in the CSF of PLWH without lympho-proliferative disorders and with no evidence of viral encephalitis. We observed that ART-naïve subjects with detectable EBV DNA in the CSF had a higher HIV RNA viral load and also higher markers of neuronal damage and inflammation; Similarly, in ART-suppressed individuals we observed increased HIV RNA and also evidence of BBB damage, greater pleocytosis and immune activation. Further studies are warranted for understanding the contribution of EBV to HIV-associated CNS disorders.

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